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METHODS USED AT WESTERN REGIONAL RESEARCH LABORATORY FOR EXTRACTION AND ANALYSIS OF PECTIC MATERIALS

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Methods used in the Western Regional Research Laboratory for the extraction, isolation, purification, and analysis of pectic substances are described in this publication. Procedures are presented for the extraction of total pectic substances and for the extraction, isolation, and purification of undegraded pectin for characterization. Specific directions are given for the determination of ammonia, moisture, ash, equivalent weight, methoxyl, anhydrouronic acid, acetyl, viscosity, jelly grade, setting time, and specific optical rotation. Methods of assay for pectinesterase (PE) and polygalacturonase (PG) are included. Later pages contain references and a list of current nomenclature of pectic substances and enzymes.

[1] (U.S. Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration
UNITED STATES DEPARTMENT OF AGRICULTURE

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Methods for Extraction and Analysis of Pectic Materials Used at Western Regional Research Laboratory

H. S. Owens, R. M. McCready, A. D. Shepherd, T. H. Schultz, E. L. Pippen, H. A. Swenson, J. C. Miers, R. F. Erlandsen, and W. D. Maclay

Pectic substances are almost universally distributed in plant tissue, where they act as cementing materials between cells, particularly in fruit tissue.[1/] Their importance in food technology can hardly be overemphasized, because they play important roles in the maintenance of texture in fresh fruits and vegetables and also in the viscosity or gel strength of processed foods like catsups, chili sauces, jellies, preserves, and low-solids gels. Problems involving pectins are continually arising. Accordingly information on the measurement, composition, and properties of pectin is essential.

Formation of jellies is impossible without pectin, and knowledge of its amount and jelly grade in the fruit juice to be gelled aids in estimation of amounts of pectin or pectin concentrate to be added.[2/] The setting time of pectin controls settling of solid particles in marmalades and jams. In fruit purees and tomato products which must have a high viscosity, the amount and properties of the dissolved pectin are important factors, but knowledge of these factors alone will not enable one to solve all the problems in this field. For example, the shape and content of fiber in purees materially contribute to their flow behavior. Pectin and other water-soluble polysaccharides on the surfaces of these fibers probably contribute to the flow properties, but this contribution has not been evaluated.

Increase of quantitative information on pectin in foods will resolve many problems in food technology. The purpose here is to provide more specific directions on methods used in this Laboratory for extraction, analysis, and evaluation of pectin, and to provide more information on analytical and physical methods than is available in the publications by Berglund (3), Hirst and Jones (19), Henglein (16), Pallmann and Deuel (44), Joslyn and Phaff (24), and Kertesz (26). The methods described here are intended to provide background information concerning pectic substances. Although these methods have practical value in problems related to texture and consistency, their use may also indicate a new approach to such problems. This field of endeavor, as it applies to woody tissue and to raw material used as a source of pectin, is under investigation by a subcommittee on pectin in the Cellulose Division of the American Chemical Society.

Extraction

It has been suggested that "a pectin determination is somewhat empirical and has in it not a little art and philosophy." An unresolved question remains whether pectic materials are present in plant tissue as protopectin and as pectate (4) or as a high-molecular-weight pectin cross-linked through cations or hydrogen bonds, or physically entrapped (15, 43, 47). Until the question receives a definite answer, "philosophy" will enter the decisions on best extraction procedures. We have assumed that pectin is present primarily as a high polymer, with a wide

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1/ Nomenclature of pectic substances is listed with definitions on page 24.

2/ The term "jelly" refers to gel systems containing not less than 65 percent soluble solids. See regulations of Food, Drug, and Cosmetic Administration.

distribution of molecular weights and some variation in methoxyl content. Except for salt linkages, no primary bonds between pectin and cellulose or other high polymer are assumed to exist. The simplest and least drastic extraction methods based on these assumptions have been chosen to obtain sufficient information to characterize pectic substances in various fruits and vegetables.

Evidence of intimate association of pectic substances with cellulose and other polysaccharides in cell walls is convincing and permits deductions concerning conditions for their extraction. First, pectin is macromolecular; therefore plant tissues from which it is to be extracted must be maintained in a swollen condition to allow its diffusion. Second, the presence of carboxyl groups in pectin offers opportunities for cross linking with other pectin molecules, proteins, and glucuronic acid groups in cellulose through polyvalent cations. Heat, acid, or cation-acceptors, or some combination of these agents, is required to disrupt these cross linkages. Third, hydrogen bridges between associated high polymers and salt linkages with proteins can be dissociated by thermal energy and addition of suitable hydrogen-bond-breaking agents.

Water was chosen as part of the extraction medium because it is a satisfactory solvent for both high-methoxyl pectins and many salts of low-methoxyl pectins and pectates. Of the extraction aids, three have been given the most attention. Acid can be used for tissue containing only small amounts of pectates; otherwise it is unsatisfactory. It is the recommended agent if information is desired on the commercial possibilities of plant tissue for jelly-type pectin. Sodium polymetaphosphate (Calgon)[3/] and sodium salt of ethylene diamine tetra-acetic acid (Versene, Regular)[3/] are the only cation acceptors considered. They form soluble complexes with polyvalent cations. Calgon, however, is difficult to remove from alcohol precipitates of pectin. Versene must be used at a relatively high pH at which pectin may be degraded. We have no reason to believe that pectins from all plant sources are the same; therefore pH stability of pectins from different plants should be checked before pH values of 6 or higher are used for the cold extractions. For total pectic substances this effect is not serious. A hot extraction method has been chosen for total pectin because of its effect on hydrogen bridges and the higher rate of diffusivity of pectin in hot solutions.

The nature of the problem and the nature and extent of information that must be obtained about the pectin *in situ* will determine how drastic the extraction procedure can be. For estimation of total pectin, extraction conditions will generally be more drastic than those required for removal of a pectin that has the molecular weight and degree of esterification of that normally present in the tissue. A number of methods have been developed to obtain maximum amounts of information about pectin in plants. Kertesz and McColloch (27) use successive extractions with water, oxalate, and acid solutions. Their results indicate that tomato pectins extracted by water and oxalate differ in degree of esterification. In apples (13) water-soluble and Calgon-extractable pectinates have about the same degree of esterification. A complete review of methods will not be given here, but only those methods will be presented that we generally use and consider most satisfactory from the present inconclusive knowledge of pectic substances.

3/ Mention of these products and others by commercial name does not constitute recommendation by the Department of Agriculture over other products not mentioned.

The methods recommended cover determination of total pectic substances and extraction of pectic substances in the cold with a metal-ion sequesterant to obtain materials for characterization. A procedure for determination of water-soluble pectin is given for its interest in certain fields. The minimum information that should be obtained on the cold-extracted, carefully precipitated, and deashed material includes percent anhydrouronic acid, percent methoxyl, percent acetyl, and intrinsic viscosity. With knowledge of amount of pectin and characteristics of cold-extractable pectin in the original and in processed tissue, conclusions can be drawn concerning the presence of pectic enzymes or whether the processing treatment was excessively drastic. Such conclusions can be drawn if the amount of material extracted in the cold is sufficient to make it representative of all the pectic substances. Only experience will enable one to set a limit for the amount of material that is typical.

Total Pectic Substances: When it is necessary to know the total amount of pectin (for example during storage tests of foods, studies on composition of foods, or estimation of recovery in commercial production of pectin) rather drastic extraction conditions are required. The method below yields results between 90 and 100 percent of those obtained by direct anhydrouronic acid analysis of grapefruit, cranberries, raspberries, and carrots and between 95 and 100 percent of results obtained by R. M. McCready and Elizabeth A. McComb of this Laboratory, whose method, unpublished at present, is more specific and involves extraction with Pectinol.3/

> Enzymes, if present, can be inactivated by treating the tissue with boiling alcohol as described on page 4. If the tissue is dried, freeze-drying or other methods which maintain a porous structure should be used, or the dried material should be ground to pass a 60-mesh or smaller screen.
>
> Weigh 100 gm. of fresh or 10 gm. of ground dried tissue into 400 ml. of water. Macerate fresh tissue thoroughly in a blender. Place the slurry in a tared container; add 1.2 gm. of freshly ground Calgon; adjust to pH 4.5, and heat slowly to about 95°C. while stirring.4/ Continue stirring and heating at 90-95° for 60 minutes. It is advisable to check the pH after 15 minutes to be sure it is at 4.5. At the same time water lost by evaporation is replaced. Do not add water during the last 20 min. of the extraction period. Add 4 gm. of filter aid and 4 gm. of ground paper pulp. Check the weight. Filter rapidly through an 11-cm. filter, using a fast filter paper coated with 3 gm. of moistened fast filter aid. Collect not less than 200 ml. Cool the filtrate as rapidly as possible.

The cooled filtrate is analyzed for its pectic content by the optical rotation, the CO_2 determination, or the carbazole method, as described later. In case reagents and equipment are lacking for these methods, the pectic substances are precipitated by alcohol as described on page 5. In any case results should be

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4/ Alternatively, the slurry is adjusted to pH 6 after adding 2 gm. of Versene. A pH of 1.8 to 2.0 without sequesterant can also be used with most tissue but is not effective when low-methoxyl pectin or pectate is present.

calculated to include the pectic substances held up in the filter pulp as well as that in the filtrate. For example, if the extract contained 300 gm. of water immediately prior to filtration (including that in the tissue) while the filtrate contained only 200 gm. of water, the amount of pectic substances in the filtrate would be multiplied by the ratio 300/200 to calculate the amount in the original tissue. This calculation saves two or more extractions.

These directions are satisfactory if the raw material is as rich in pectin as citrus peel (3 percent). Most sources require a larger sample and a lower ratio of water to sample. With fruits a 2-to-1 ratio will be better than the 4-to-1 given above. If the carbazole procedure is used for determination of the uronide content, all quantities can be greatly reduced so that operation can be carried out in centrifuge tubes.

Pectin obtained by this method will be somewhat degraded but is satisfactory for laboratory exercises on the properties of pectin. Its properties are a rough indication of the care with which the source material was treated.

"Water-Soluble" Pectin: Problems concerning viscosity of purees, tomato products, and jellies require information on the amount and nature of pectin liberated during processing and some knowledge of fiber content and cations present in the medium. Usually addition of 1 percent of filter aid and 1 percent of ground paper pulp will permit pressing or filtering the juice. Isolation of the pectin present is accomplished by directions given on page 5. Yields of this material are not significant unless particle size is controlled.

Cold-Extracted Pectin: To supplement information available from the amount and nature of the so-called "water-soluble" pectin, it is desirable to isolate pectin which is comparable to that *in situ*. Pectic enzymes must be inactivated. This is accomplished by slicing the tissue into 95 percent ethanol above 70°C. The final ethanol concentration should be not less than 70 percent. The heating period should be about 12 to 18 minutes. To maintain a porous structure and to prevent possible loss of pectin by reaction with other compounds, it is advisable to store the alcohol-treated tissue either under alcohol or in the frozen state. If it is dried, a freeze-drying or other vacuum drying technique should be used. It will be noted that methods of pretreating the sample to obtain a pectin representative of that *in situ* are much less drastic than those for total pectin. We have found that prolonged heat treating and drying of samples at high temperatures are unsatisfactory if a high-molecular-weight pectin is desired. The procedure follows:

> Blend 100 gm. of tissue (previously treated with alcohol) with 200 gm. of water. Add 2 gm. of Calgon or Versene. Adjust the pH to 4.5 for the phosphate or 6 for the amine derivative. After 24 hours at room temperature, with occasional stirring, add 3 gm. of filter aid and 3 gm. of paper pulp, heat to 60°C., and separate the solution by filtration, centrifuging, or pressing. Accomplish isolation as described under that section except that 1 volume, or between 1 and 2 volumes, of alcohol or acetone containing acid are used for the precipitation. Two precipitations are recommended, followed by washes with neutral 70 percent solvent and finally pure solvent. If it is desired to remove 80 percent or more of the pectin, a second extraction is recommended. Toluene or other antiseptic should be used during these extractions.

Cold-extracted pectin is prepared primarily to study properties of a pectin that is comparable to that *in situ*. It should be carefully de-ashed, freed of excess acid, and dried at no more than 60°C. *in vacuo*.

Comparisons between cold-extracted and water-soluble pectin should be made in a sufficient number of cases to determine whether or not they are the same. Pectin is located in and between the cell walls and possibly is out of contact with pectic enzymes until the cells are ruptured. The pectin in the juice might be the result of leaching of the membranes near cell ruptures and thus would be typical of *in situ* pectin. Cold sequesterants decrease the number of salt linkages involving pectin, facilitating removal of an undegraded pectin.

Isolation and Purification: Pour the cooled, weighed, or measured filtrate into 3 volumes of ethanol, 2-propanol, or acetone containing 0.5M concentration of HCl. (The pH of the slurry should be between 0.7 and 1.0.) Stir for one-half hour. Centrifuge, filter, or separate the precipitate on coarse-mesh nylon (bolting cloth). A second wash at the same pH is recommended to remove all but traces of ash. Wash repeatedly in 400-ml. portions of 70 percent alcohol or acetone until the precipitate is essentially chloride ion-free or the pH is above 4. Dehydrate the precipitate further in 400 ml. of acetone. Dry overnight *in vacuo* (5 mm. Hg pressure) with a slow stream of dry air passing through the oven. Weigh the precipitate. Determination of methoxyl, ash, and uronide content will yield information on the total amount of pectin. Until more is known about the composition of pectin, the uronide content will be considered a measure of the total yield of pectin. Pectin yields based on the alcohol precipitate will be higher by 15 to 50 percent than the uronide values.

If acid is not used in the organic precipitant, the correction for ash will not be reliable (see Ash Determination).

Acetone is used in the final step before drying to remove any alcohol that might be sorbed by the precipitate, since such alcohol would interfere with Zeisel determination for methoxyl (20).

If the concentration of pectin is below 0.2 percent in the filtrate, it should be concentrated to that level *in vacuo* before precipitation in alcohol.

Qualitative Tests

Pectic Substances: A useful qualitative test for the presence of pectic substances is a modification of Ehrlich's lead acetate test (14).

Dissolve or suspend the material under question in water at approximately 1 percent concentration of substance to be tested and add 0.2 percent of potassium oxalate and 0.1 percent of Pectinol. Mix and cover with toluene and allow it to stand for 20 or more hours. Remove the solids by filtration or centrifugation and test the solution for galacturonic acid. Add saturated basic lead acetate solution to 5 ml. of solution. If galacturonic acid is present, a white precipitate

forms and re-dissolves upon addition of excess or an equal volume of the lead reagent. Boil the mixture. Formation of a red precipitate is a positive test for galacturonic acid and therefore of pectin in the original substance.

Ammonia: The presence of ammonia will interfere with many of the proposed analytical methods.

Add 1 ml. of 0.1N NaOH to a small amount of dried sample. On heating, the presence of ammonia can be detected by its odor or, better, by moistened litmus paper. Wash out ammonium ion with acidified 60 percent alcohol, followed by neutral alcohol to remove acid.

Quantitative Analysis

Pectin samples prepared by the foregoing procedure will be dry and should be stored under dry, cool conditions. In moist climates, however, weighing dry pectin samples is difficult because they rapidly adsorb water vapor. Under these conditions samples should be exposed to the air of the laboratory for 1 or 2 days until they reach an equilibrium moisture level. The moisture content is then determined and a correction made for it on all other analyses and physical measurements. Results of analyses and physical measurements should be expressed on a moisture-, ammonia-, and ash-free basis.

Ash-free low-methoxyl pectins and pectic acid are only slightly soluble in water. To prepare solutions of them it is necessary to exercise care in adding dilute alkali to the rapidly stirred suspension to avoid degradation or deesterification. Such samples should dissolve when the pH has been increased to 4 or 4.5.

Commercial pectins are likely to contain reducing sugars which can be removed by washing with 60 percent alcohol. The final wash should be with acetone.

Ammonia: Ammonia is determined quantitatively in pectic substances by distillation from magnesium oxide suspension at pH 10.9. Total nitrogen is determined quantitatively by the conventional Kjeldahl procedure.

Moisture: Weigh 1 gm. of sample ground to pass 80-mesh into a tared moisture dish (metal, 2 in. in diameter with cover). Dry *in vacuo* (5 to 20 mm. of Hg) for 4 hours at 100°C. Place in a desiccator and cool to room temperature and weigh. Desiccant should be phosphorus pentoxide or similar effective agent. The pectin is degraded by the process and should not be used for subsequent measurements. If the sample is to be used for other measurements, drying should be done at 70°C. for 16 hours. One percent should be added to the percent moisture to obtain agreement with the Fischer method (22).

Ash: Weigh 1 to 2 gm. of pectic substance ground to pass 80-mesh into a tared crucible. Ignite slowly, then heat 3 or 4 hours at 600°C. Cool the crucible to room temperature in a desiccator and weigh. Determine the alkalinity of the ash as follows. Dissolve the ash in an excess of 0.1N HCl, 25.00 ml. usually being sufficient. Heat gently

to boiling and after cooling, titrate with 0.1N NaOH, using phenolthalein as an indicator. Calculate the basicity in terms of carbonate and express the results as ash minus carbonate.

A number of errors are likely to occur in the determination of ash. Volatile impurities such as ammonium salts will give low results. Some cations, notably aluminum, iron, and others, form insoluble oxides which will not be titrated in the determination. It is better to de-ash the pectin with acidified alcohol or ion exchange materials as previously described, so that little or no correction for ash is needed.

Equivalent Weight: Values for equivalent weight are used in the calculations for anhydrouronic acid content and degree of esterification.

Weigh 0.5 gm. of pectic substance into a 250-ml. titration flask and moisten with 5 ml. of ethanol. One gram of NaCl may be added to sharpen the end point. Add 100 ml. of carbon dioxide-free distilled water and 6 drops of phenol red indicator (or Hinton's indicator (17)--bromothymol blue 0.4 percent 1 volume, phenol red 0.4 percent 3 volumes, cresol red 0.4 percent 1 volume, and distilled water 1 volume). Slowly titrate to avoid possible deesterification, using 0.1N NaOH until the indicator changes (pH 7.5). Be sure all of the pectic substance has dissolved and that no lumping on the sides of the flask occurs. The endpoint should persist for 30 seconds. Save the neutralized solution for methoxyl determination.

$$\text{Eq. wt.} = \frac{1000 \times \text{wt. of sample (gm.)}}{\text{N} \times \text{vol. of alkali (ml.)}}$$

Ammonia- and ash-free pectinic or pectic acids should be used for this determination. Corrections can be made for such constituents, but the results are usually not completely reliable.

Methoxyl Content: The methoxyl content or degree of esterification is an important factor in controlling the setting time of pectins, the sensitivity to polyvalent cations, and their usefulness in low-solids gels, films, and fibers.

To the neutral solution titrated for equivalent weight containing 0.5 gm. of pectic substance, add 25 ml. of 0.25N NaOH, shake thoroughly, and allow to stand 30 minutes at room temperature in a stoppered flask. Add 25 ml. of 0.25N HCl (or an amount equivalent to the base added) and titrate with 0.1N NaOH to the same endpoint as before (17).

$$\% \text{ MeO} = \frac{\text{N} \times \text{vol. of alkali (ml.)} \times 3.1}{\text{wt. of sample (gm.)}}$$

The Zeisel method for alkoxyl as modified by Shriner (49) offers a precise means of determining the methoxyl content and uses small amounts of sample. Sorbed methanol, ethanol, or 2-propanol must be washed out with acetone or removed by humidification. Presence of acetyl does not interfere, but glycosidic and ether methoxyl groups are measured in the same manner as ester methoxyl. The procedure follows:

Weigh 15 to 50 mg. of pectic substance (depending on the methoxyl content) into a small glass container. Place the sample plus a boiling chip in the reaction flask and add 2 ml. of melted phenol and 5 ml. of 48 percent HI. Connect the flask to the remainder of the apparatus, which consists of an air condenser, a scrubber, and a receiver. The scrubber contains 5 percent solutions in equal amounts of sodium thiosulfate and cadmium sulfate. The receivers contain 10 ml. of acetic acid, 1 gm. of potassium acetate, and 15 drops of bromine. Bubble a slow stream of carbon dioxide through the reaction flask and boil the liquid gently with a micro burner. The vapors should condense within 8 cm. from the bend of the air condenser. After 30 minutes, wash the contents of the receivers into a 300-ml. flask with 100 to 150 ml. of water. Add 1 to 1.5 gm. of sodium acetate hydrate and after solution is complete add formic acid dropwise while shaking the flask to destroy excess bromine. After the bromine is destroyed add 1 gm. of KI, 10 ml. of concentrated HCl, and titrate the liberated iodine with 0.1N sodium thiosulfate using starch as an indicator. The calculation is made from the following:

$$\% \text{ MeO} = \frac{517 \times N \times \text{vol. of } Na_2S_2O_3 \text{ (ml.)}}{\text{wt. of sample (mg.)}}$$

When the purity of a pectin sample is to be calculated, reduce the methoxyl ($-OCH_3$) to methylene ($-CH_2$), since the anhydrouronic acid analyses include the weight of this oxygen atom in the formula weight of 176.

Anhydrouronic Acid: Although pectin is sometimes considered a partly esterified polygalacturonide, , it actually contains 10 percent or more of unaccounted-for organic material, which is composed of arabinose, galactose, and perhaps other sugars. Information on the percentage of anhydrouronic acid is necessary to establish purity and degree of esterification and to evaluate the physical properties.

If the equivalent weight, methoxyl content, and alkalinity of the ash have been determined the calculation is as follows:

$$Z = \frac{\text{wt. of sample (mg.)}}{\text{m.e. alkali for free acid + m.e. alkali for saponification + m.e. titratable ash}}$$

$$\% \text{ A.U.A.} = \frac{176 \times 100}{Z}$$

A second method for determining the anhydrouronic acid content, which gives the same results as titration, is a modification (35) of the Lefevre-Tollens (29) method. Carbonates interfere but ash and ammonia do not, because this method depends on decarboxylation of the uronide. The procedure follows:

Weigh about 250 mg. of pectic substance into the 125-ml. reaction flask and add 30 ml. of 19 percent HCl. Sweep the apparatus with carbon-dioxide-free air and then attach the absorption tower. Add 25 ml. of 0.25N NaOH and a few drops of n-butanol to the absorption tower. Immerse the reaction flask in an oil bath and raise the temperature of the bath to 145°C. while sweeping the apparatus with a stream of

carbon-dioxide-free air for a heating period of 1.5 to 2 hours. Disconnect the absorption tower, wash the contents into a 300-ml. flask, and add 10 ml. of 10 percent barium chloride solution. Titrate the excess alkali, using 0.1N HCl until just colorless to phenolphthalein. The value so obtained, expressed in ml., subtracted from the volume (ml.) of 0.1N HCl required for titration of a control standardization of 25.0 ml. of 0.25N NaOH is the net volume of acid equivalent to the carbon dioxide. The calculation is carried out by the equation

$$\% \text{ A.U.A.} = \frac{\text{N} \times \text{net ml. of acid} \times 8.8}{\text{wt. of sample (g.)}}$$

Stark's (51) method for the microdetermination of pectic substances in cotton based upon Dische's (12) carbazole reaction has been modified (34) as a result of a critical study of the variables affecting the quantitative aspects of the reaction. The modified method shows promise for the determination of total pectic substances.

Deesterify a 0.1 percent solution of pectin in 0.05N NaOH for 30 minutes at 25°C. and dilute to 0.002 percent. Add 2 ml. of this solution to 12 ml. of ice-cold concentrated sulfuric acid in a 25x180-mm. test tube. Mix and heat the tube and contents in a boiling-water bath for 10 minutes. Cool the tube and contents to 20°C. and add 1 ml. of ethanol containing 1.5 mg. of carbazole. Mix thoroughly and after 25±5 minutes at room temperature, determine the color intensity with light of wave length 520 mμ. A blank containing the sample plus reagents (without carbazole) is used to set the colorimeter. A standard curve with 20 and 40 micrograms of galacturonic acid hydrate (mol. wt. 212) is used to obtain the anhydrouronic acid content (mol. wt. 176) of the pectic substance analyzed. Hexose sugars in two-fold excess, glucuronic acid, and mannuronic acid interfere.

Acetyl: Sugar-beet pectin and perhaps others contain acetyl groups. Analysis for this functional group by simple alkaline saponification procedures followed by back titration does not yield satisfactory results. Clark's (9) method was therefore modified to be applicable to pectic substances (46). The procedure follows:

Weigh 0.5 gm. of pectin into a 250-ml. Erlenmeyer flask and add 25 ml. of 0.1N NaOH. Stopper the flask and stir the contents until the pectin is dissolved. Set the flask aside for at least 1 hour (overnight is also permissible), dilute the contents to 50.0 ml., and withdraw a 20.0-ml. aliquot. Place the aliquot in the distillation apparatus. Add 20 ml. of magnesium sulfate-sulfuric acid solution (100 gm. magnesium sulfate (crystal) and 1.5 gm. of sulfuric acid diluted to 180 ml.). Steam distill and collect about 100 ml. of distillate, keeping the volume in the distillation flask low. Titrate the acetic acid with 0.05N NaOH to a phenol red endpoint. A blank titration on water plus 20 ml. of the magnesium sulfate-sulfuric acid distilled as described amounts to less than 0.1 ml. of the standard alkali. The calculation is made as follows:

$$\% \text{ acetyl } (-\text{OCCH}_3) = \frac{\text{N} \times \text{vol. of alkali (ml.)} \times 4.3}{\text{wt. of sample in aliquot (gm.)}}$$

Viscosity: The contribution that pectin makes to jelly formation, to the viscosity of many food products, and to the firmness of texture is due in part to its molecular weight. The simplest measure of viscosity average molecular weight is the intrinsic viscosity (V_i). This is defined as the limiting value for the ratio $\frac{\eta_r - 1}{C}$ as C, the concentration, approaches zero, where η_r is the viscosity of the solution relative to the solvent. The intrinsic viscosity is indicated as $[\eta]$, η_i, or, in this report, V_i.

Pectin contains carboxyl groups, which, when ionized, contribute to the flow behavior of pectin solutions, because electrostatic repulsion between them cause lengthening of the chain. This effect can be reduced by addition of either salt or acid. The procedure follows:

> Weigh exactly 0.1 gm. of pectic substance (ash- and moisture-free basis). Dissolve in 50 ml. of water, adjusting the pH if necessary, Stir two hours. Add 0.80 gm. of sodium chloride and 0.200 gm. of Calgon or neutral Versene in 15 ml. of distilled water and stir another hour. Adjust the pH to 6.0±0.2. Rinse the electrodes into the solution and make up to 100.46 gm. or transfer to a 100-ml. volumetric flask and make to volume. Stir rapidly and thoroughly one minute and cover or stopper. If the solution is cloudy or contains dust or fibers, centrifuge in covered tubes or pass through a coarse sintered-glass filter. Determine the viscosity of the solution within an hour after the pH adjustment, by means of an Ostwald-Cannon-Fenske No. 50 pipet with 10 ml. of solution at 25±0.03°C. (A Hoeppler falling-ball type of viscometer can be used as an alternative method.) Determine the efflux time in the same instrument for the solvent, which is considered to be 0.8 percent sodium chloride, 0.2 percent Calgon solution. Because the density difference between solution and solvent is only 4 parts in 10,000, the relative viscosity is essentially equal to the ratio of the time of efflux for solution to that for solvent. Obtain the intrinsic viscosity directly from the curve in Fig. 1, which is based on Martin's exponential equation, in which the constant, k', is assumed to be 0.40 (40).

Maximum errors in intrinsic viscosity resulting from deviations in k' (K_m) with citrus or apple pectic substances are not more than 0.06 at V_i = 3.5 and 0.20 at V_i = 7.0. If greater accuracy is required, determine the relative viscosity at three concentrations, such as 0.15, 0.10, and 0.05 gm. per 100 ml., plot the ratio $(\eta_r - 1)/c$ against c on semi-log paper, and extrapolate to zero concentration to get the intrinsic viscosity.

Care should be exercized in cleaning the viscometer. When in doubt, cleaning solution should be used. When a series of determinations is made on the same day, thorough rinsing five times with distilled water followed by a pure 95 percent ethanol or acetone rinse and suction drying is sufficient.

The degree of esterification influences the value of the intrinsic viscosity. Our results agree with the conclusion of Vollmert (53) that the intrinsic viscosity decreases with increasing esterification. The magnitude of this influence is still under investigation. Inasmuch as most comparisons are made between pectins of similar ester content, viscosity can be a useful index of jelly grade or molecular

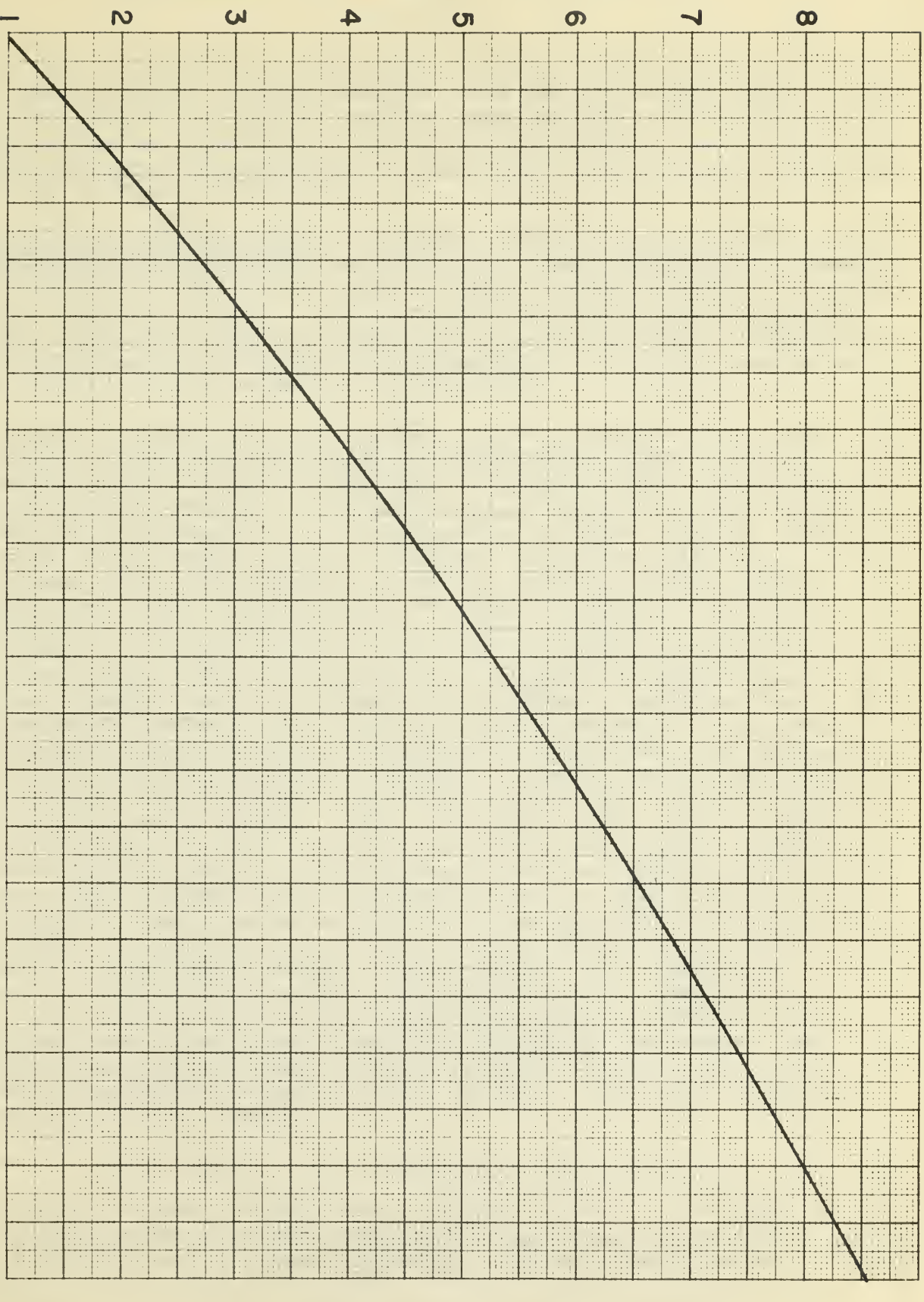

INTRINSIC VISCOSITY, DL. PER G.
1
2
3
4
5
6
7
8
RELATIVE VISCOSITY OF 0.100% W/V SOLUTION
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.1
2.2

FIGURE 1

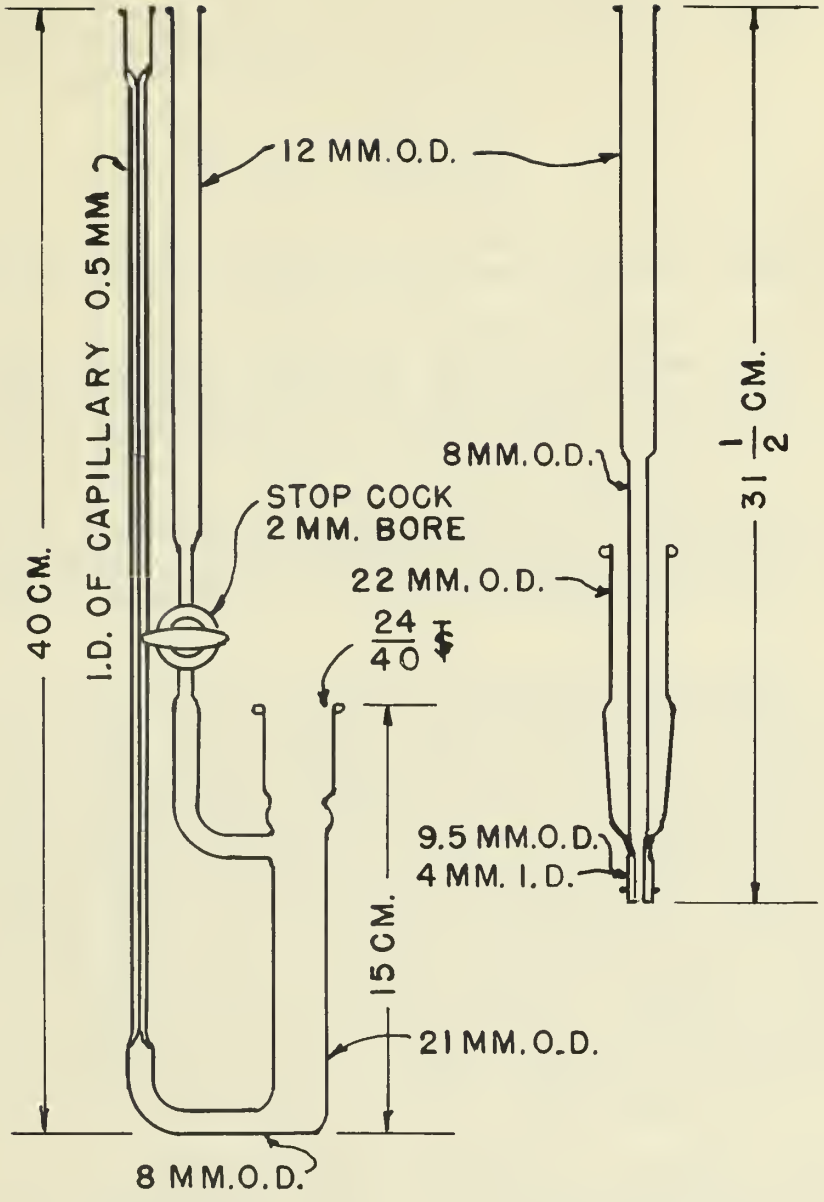
weight. Do not deesterify by alkali and measure the intrinsic viscosities of pectic acid in order to compare molecular weights of pectins (53, 54). The beta-elimination hypothesis indicates that the higher the degree of esterification the greater will be the degradation of pectin with alkali (54). Pure pectinesterase might be used for this purpose, but the time required for complete reaction and subsequent purification is excessive.

Number Average Molecular Weight. The number average molecular weight is an important factor controlling jellying with pectin. Many investigators recommend converting pectin to the nitrate, then measuring intrinsic viscosity in acetone solution, and relating that to molecular weight measured by osmotic pressure (16, 50). Direct measurement is preferable to reduce the chance of degradation and of fractionation. We use the Bull osmometer (5, 6) slightly modified as shown in Fig. 2. Instructions for its use follow:

> Prepare solutions of pectic substances at three concentrations, preferably below 0.60 percent (ash- and moisture-free basis), in 0.2M sodium chloride at pH 4.5. Solutions are preserved with 0.001 percent phenylmercuric nitrate. Introduce 0.5 ml. of toluene into the capillary of the osmometer. Fill the chamber of section A with salt solution. Knot a 5-in. section of water-swollen uncoated cellulose sausage casing (wall thickness 20 microns, diameter, 12/32, 18/32, or 8/32 in.). Trim to proper length and tightly fasten to lower end of section B with a rubber band or linen thread. Test sac by immersion in water, and application of air pressure through top of section B. Remove excess water, rinse inside of sac with pectin solution, and fill to desired height, removing all air bubbles. The difference in levels in sections A and B should be near the estimated osmotic pressure of the pectin solution. It is helpful to mark these two levels on the tubes with wax pencil in advance. Grease joints with stopcock lubricant. Join sections A and B with stopcock open. Restore shape of sac and remove bubbles, if any, from region of stopcock by blowing on section B. Add salt solution to section A to the desired level. Remove any bubbles from toluene column by suction. Retard evaporation from tubes by covering them with small vials or slotted corks or stoppers. Immerse osmometer in water bath at 25° ±0.01°C. After 30 minutes read liquid levels in all three arms by means of a cathetometer or traveling telescope. Close stopcock. Read toluene and pectin solution levels every day until difference is constant. At least 5 days are required with commercially available tubing. (Before reading the toluene level it should be temporarily raised about 1 mm. by gentle suction. If the capillary is clean, the level will return to its undisturbed position in a few seconds.) Calculate the osmotic pressure from the equation:

$$P = 1.01 (S_1 - B_1) + 0.866 (T_1 - T_2) - (S_1 - S_2)$$

> where P is osmotic pressure in cm. of water at 4°C.; S_1 is initial level of pectin solution; B_1 is initial level of salt solution; T_1 is initial level of toluene; subscript 2 refers to final levels; 1.01 is the density of the pectin in sodium chloride solution as described above. Divide osmotic pressure by pectin concentration



MODIFIED BULL OSMOMETER

FIGURE 2

expressed as percentage (gm. per 100 ml. of solution) and plot P/C vs. C. The intercept at C = 0 is the limiting value for the ratio P/C and is used in the calculation of molecular weight. If temperature during measurement is maintained at 25°C., the molecular weight is calculated from the equation:

$$M_{No.\ av.} = \frac{25.3 \times 10^4}{(P/C)_{C \to 0}}$$

Significant diffusion of pectin through the membrane or leaking past it can be detectedby testing the salt solution by means of carbazole (51) (page 9.

The osmometer must be clean so that capillarity differences will not introduce errors. If aqueous solution menisci do not have the same shape or if the toluene does not return to the same level after being raised repeatedly about 0.2 cm., clean the osmometer with "cleaning solution" before the next determination.

It is advisable to measure duplicates on each concentration. If $(S_1 - B_1)$ is set a little above the expected osmotic pressure in one determination and below it in another, a plot of apparent osmotic pressure vs. time will increase the accuracy and enable determination of the equilibrium value in a shorter period of time.

Jelly Grade: The most important direct application of pectin is as a gelling agent. To know how much pectin to add to a fruit juice to make a satisfactory jelly, the jelly grade must be known. This is defined as the number of grams (or lbs.) of sugar with which one gram (or lb.) of pectin will form a 65 percent soluble-solids gel of specified strength under suitable acid conditions. No standard amount of acid nor strength value has been accepted; consequently there is likely to be variation in jelly grades of pectins purchased from different companies. Various methods for jelly-grade determination have been developed. The English use the B A R tester, devised by Campbell (7). The Germans use the Lüers-Lochmüller tester (31). In this country the Tarr-Baker tester (52) and the ridgelimeter (11) have met with favorable response. Methods of grading pectin are under examination by a committee of the Institute of Food Technologists with the objective of establishing a standard method. Methods of preparing jellies vary considerably and only a modification of the simplest (11) will be described

Weigh 5.20 gm. of pectin (assume 150-grade as a trial), mix with 50 gm. of sugar and dissolve in 400 ml. of water. If the pectin does not dissolve, add a small amount of Calgon. Adjust the pH to 3.2 + 0.1. Pour into a tared jelly pan (2 qt.), adjust net weight to 525 gm., adding water rinsings of the beaker which contained the pectin. Bring to a boil in less than 5 minutes, with stirring. Start timer and slowly add 725 gm. of sugar with stirring. Stir and boil for 2 minutes after addition of last portion of sugar to insure complete solution of pectin. Total time of heating at or near boiling should not exceed 5 minutes. At 4 minutes and 30 seconds the weight should be checked and the net weight brought to 1200 gm. either by evaporating more water or adding some. Bring to boiling again and at the end of 5 minutes pour the solution into jelly glasses. The glasses have a known depth (7.95 cm. high inside 11, 23) and should contain

2 ml. of acid solution (1 gm. citric acid monohydrate to 1 ml. of water) when the pectin solution is poured. The glasses should have sideboards 3/4 in. above edge of glass. Scotch or drafting tape can be used for this purpose.

Stir contents of the glasses vigorously for 1 or 2 seconds and set aside for 24 hours. Slice off the jelly flush with top of glass. Remove jelly from glass and place it bottom side up on a clean, dry platform of the ridgelimeter. Measure the percentage of sag after 2 minutes and read the deviation from assumed grade (23). If the assumed grade was off by more than 20 percent, make another jelly closer to specified strength. The pH of the jelly should be 2.4 or less.

The torsion tester yields the same results (42) as does the B A R tester (38) and offers the advantage of yielding fundamental values if a cylinder is used instead of a blade. The ridgelimeter can be used to obtain modulus of elasticity in compression if strong jellies are cast in the form of cylinders.

Jelly grade can be determined from the intrinsic viscosity, by means of the curve shown in Fig. 3. Further work will be necessary to establish its reliability, because it represents data from only a few citrus pectins, and molecular weight heterogeneity probably has a marked influence on the curve.

Points that should be mentioned: The pectin must be completely dissolved before heating. Any pectin precipitated by the added sugar should be redissolved before the gelling solution is poured into glasses. The temperature of storage should be near 23° to 25°C., especially with slow-set pectins (38). Rate of application of force should be constant. Only small displacements should be applied for calculations of elastic moduli. One advantage of the sag tester and rigidometer is that the force is applied for a constant period of time for each jelly. Displacements measured under these conditions are more likely to yield fundamental constants than a breaking test in which force must be applied until breaking results. The latter, of course, causes more yielding of the gel structure. One result is that grades measured by the two types of testers seldom agree.

It has been stated (8) that it is unsound to measure fundamental constants of pectin jellies. Such measurements have been made even on solutions of polymers by applying force over very short periods of time (1). Similarly if displacements of pectin gels are small, they are also essentially linear with respect to applied force (37, 41), and calculations of elastic moduli are reliable. The arguments and data offered by Cheftel and Mocquard (8) indicate that breaking strength measurements are not reliable; on the other hand Bender (2), by carefully controlling each step, reports that the Tarr-Baker tester gives reproducible results.

No mention has been made of grading low-methoxyl pectins. We generally measure methoxyl content and intrinsic viscosities to establish a value for comparison of such pectinic acids. Generally a pectin prepared by the method used in this Laboratory (41), with a uniform methoxyl content of 3.2 ± 0.3 percent and an intrinsic viscosity of 3.4 ± 0.5, will be satisfactory for gel purposes. For films and fibers the lower limit for viscosity is about 3.5. Higher values of methoxyl content require additional calcium ion for gelation, while higher values of viscosity require less calcium. By varying calcium ion concentration, pectinic acids outside the mentioned range can be applied for formation of gels, etc.

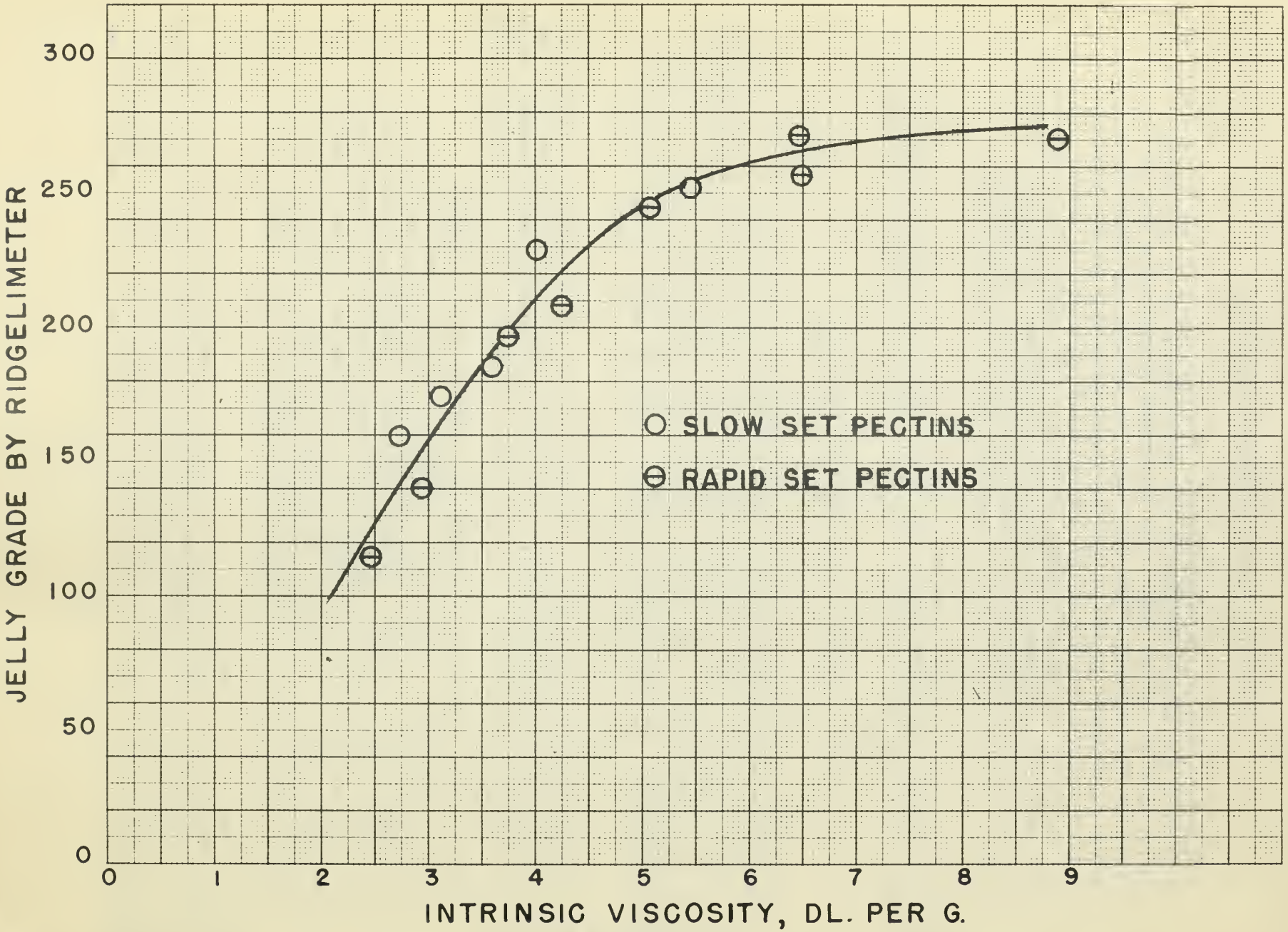

JELLY GRADE BY RIDGELIMETER
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
9
INTRINSIC VISCOSITY, DL. PER G.
SLOW SET PECTINS
RAPID SET PECTINS

FIGURE 3

Setting Time: The setting time of pectins is of importance to jam, marmalade, and preserve processors. Several methods have been proposed to measure this factor (18, 23, 39). Rate of cooling, presence of metal ions, pH, solids content, etc. influence setting time and temperature (18, 41). We have selected, but not tested, a simple method (23), which involves no change in technique from that used for preparing jellies for grading.

> Prepare jelly as before and start a timer when filling a sample glass. Place the glass in a bath maintained at 30°C. so that the glass is surrounded almost to the top. At intervals give it a slow twist. When the jelly at the top just congeals, stop the timer to obtain the setting time. Gelation can be observed by slowly turning the glass. When the turning motion ceases, a liquid system will continue to rotate in the same direction. A gel system will show elasticity and move in the opposite direction. Rapid-setting pectins will gel in less than 2 minutes, while slow-setting pectins require more than 3 minutes.

Usually a high-molecular-weight pectin with 10.5 percent or more ofmethoxyl groups (degree of esterification (DE) above 70 percent) will be a rapid-set pectin; those with 7 to 10 percent methoxyl (DE 50 to 70 percent) will be slow-set pectins. At lower DE's the pectin will tend to be rapid-set; also the presence of polyvalent cations drastically decreases the setting time.

Optical Rotation: The rotation of polarized light by pectin solutions is a characteristic property which can be used to determine pectin when the specific rotation is known (36). This value is measured by dissolving 0.5 gm. of pectin in 75 ml. of water, adjusting the pH to 4.5 to 7.0, and making to 100 ml. or gm. The solution is poured into a 2-dm. tube, and the optical rotation is measured in a polarimeter or saccharimeter at 25°C. With a saccharimeter which has a Bates-Jackson scale, the specific rotation is obtained by the equation,

$$[\alpha] = \frac{\text{obs. rot. x 100 x 0.346}}{2 \text{ x } C}$$

Pectin from oranges has a specific rotation of +230 when the methoxyl content is about 10 percent. To apply this to the determination of pectin in extracts of orange peel, the following procedure is used.

> Filter 100 ml. of the solution containing about 0.5 percent pectin with filter aid, discarding the first 25 ml. Measure the optical rotation of the filtrate in a 1-dm. tube. To 25 ml. of this solution add 25 ml. of copper sulfate solution. (The copper sulfate solution contains 9.4 gm. of the pentahydrate, 27.2 gm. of sodium acetate trihydrate, 12 ml. of glacial acetic acid made to one liter.) Filter the precipitated copper pectinate and measure the rotation of the filtrate in a 2-dm. tube. The difference between this and the rotation of the pectin extract is the net rotation due to the pectin alone and is used in the calculation.

$$\% \text{ pectin} = \frac{\text{net rotation x 0.346 x 100}}{230}$$

Yields by this method are higher than those by uronide determination.

Pectic Enzymes[5/]

Changes in the nature of pectin will occur in unblanched purees, macerated, or broken tissue through action of pectic enzymes present. These changes occur even in the frozen state. It should be mentioned that the enzymes increase in activity with increase in temperature; therefore during inactivation by heat there is a period when pectic enzymes are very active. Pectinesterase deesterifies pectin, making it susceptible to precipitation by calcium or other polyvalent cation. Juice clouds settle and frozen orange concentrates gel. Pectinases degrade pectin to galacturonic acid or low-molecular-weight polymers of it. Viscosity changes occur and in this case, too, juice clouds will settle. Measurement of the activities of these two types of enzymes provides valuable clues to the solution of many problems involving gelling, viscosity changes, syneresis, and cloud instability. The methods for such measurements follow:

Pectinesterase (30) (Pectase, Pectin Methylesterase): Pectinesterase in most plant tissues is rarely in solution but is adsorbed on the insoluble cellular solids. It is then necessary to extract this material in 0.25M NaCl and maintain a pH of 8 for about 1 hour while the enzyme dissolves. The assay for its activity is carried out as described (32).

> Add 2 ml. of 1.5M NaCl to 10 ml. of 1 percent pectin solution, stir with carbon dioxide-free air and titrate to pH 7.5 with 0.02N NaOH. Use a constant-temperature bath to maintain a temperature of 30°C. and add enzyme and water to adjust the volume to 20 ml. Immediately record the time and volume of alkali required to maintain the pH at the constant value. Adjust the concentration of enzyme to require about 1 to 3 ml. of 0.02N alkali in 10 minutes. The results can be expressed in pectinesterase units (PE.u./gm., the expression for milliequivalents of ester hydrolyzed per minute per gram of enzyme).

Polygalacturonase (PG): Polygalacturonase (sometimes considered synonymous with pectinase) seldom occurs in higher plants, and then only in small amounts. It is produced by many bacteria and fungi and has been reported in snails. Little information exists on extraction of the enzyme from plants, but its assay can be conducted on plant extracts or macerates as described. Although a quantitative method, based on viscosity changes, would be more desirable, our experience has been based on changes of reducing value as presented below (21).

> Add 1 ml. of enzyme solution of the proper dilution to 99 ml. of 0.5 percent solution of pectic acid at pH 4 and at 25°C. Remove aliquots of 5 ml. and add to 0.9 ml. of 1M sodium carbonate in a glass-stoppered flask. Add 5 ml. of 0.1N iodine, stir thoroughly, and after 20 minutes acidify with 2 ml. of 2M sulfuric acid and titrate the residual iodine with 0.05N sodium thiosulfate solution. A calibration curve prepared with galacturonic acid monohydrate is used to obtain the activity. Under these conditions, 1 milliequivalent of reduced iodine corresponds to 0.513 millimole of aldose liberated. The activity is conveniently expressed as (PG.u.)/ml., or the millimoles of reducing groups liberated per minute per milliliter of enzyme.

5/ Nomenclature is listed on page 24.

Other Pectic Enzymes: Other pectic enzymes are mentioned in the literature (28, 30, 45, 48), but for the most part they are not so well characterized as PE and PG; consequently no method of assay is recommended for them. The depolymerase activity discovered by McCulloch and Kertesz (33) can be measured in the same way as PG activity with pH adjusted to 4.5. The polymethylgalacturonase activity described by Seegmiller and Jansen (48) was also measured in the same way as PG, except that citrus pectin at pH 6.0 was used.

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Nomenclature

The American Chemical Society has recommended the following nomenclature (25):

Pectic Substances. "Pectic substances" is a term applied as group designation for those complex colloidal carbohydrate derivatives which occur in or are prepared from plants and contain a large proportion of anhydro-galacturonic acid units which are thought to exist in a chainlike combination. The carboxyl groups of polygalacturonic acids can be partly esterified by methyl groups and partly or completely neutralized by one or more bases.

Protopectin. The term "protopectin" is applied to the water-insoluble parent pectic substances which occur in plants and which upon restricted hydrolysis yield pectin or pectinic acids.

Pectinic Acids. The term "pectinic acids" is used for colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups. Pectinic acids under suitable conditions are capable of forming gels with sugar and acid or, if suitably low in methoxyl content, with certain metallic ions. The salts of pectinic acids are either normal or acid pectinates.

Pectin. The general term "pectin" (or pectins) designates those water-soluble pectinic acids of varying methyl ester content and degree of neutralization which are capable of forming gels with sugar and acid under suitable conditions.

Pectic Acids. The term "pectic acids" is applied to pectic substances mostly composed of colloidal polygalacturonic acids and essentially free from methyl ester groups. The salts of pectic acids are either normal or acid pectates.

The Committee on Nomenclature is acutely aware of the difficulties connected with the task. They stressed the fact that pectic materials represent mixtures of polygalacturonides with variable molecular weights and, with the exception of pectic acid, may also contain different proportions of methyl ester groups in the individual polygalacturonic acids. Acid salts may contain polymer units with different proportions of metallic ions, and sometimes several species of cations. These complexities must be borne in mind when solutions to pectin problems are sought.

Pectic Enzymes. The nomenclature for pectic enzymes has not been changed since 1926 (10), but a recently proposed nomenclature defines them as follows (30):

Protopectinase is a name used for the enzyme that converts protopectin into a soluble product; other names are pectosinase and propectinase. The existence of protopectin or protopectinase is not firmly established.

Pectinesterase (PE) is the enzyme that catalyzes the hydrolysis of the ester bonds of pectic substances to methanol and pectic or pectinic acids; other names used are pectase, pectin demethoxylase, pectin methoxylase, pectolipase, and pectin methylesterase.

Polygalacturonase (PG) is the enzyme that catalyzes the hydrolysis of glycosidic bonds between deesterified galacturonide residues in pectic substances; other names are pectinase and pectin glycosidase.